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Histopathological and biochemical comparisons of the protective effects of amifostine and L-carnitine against radiation-induced acute testicular toxicity in rats

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Summary

The aim of this study was to compare the radioprotective efficacies of amifostine (AMI) and L-carnitine (LC) against radiation-induced acute testicular damage. Thirty Wistar albino rats were randomly assigned to four groups: control (n = 6), AMI plus radiotherapy (RT) (n = 8), LC plus RT (n = 8) and RT group (n = 8). The rats were irradiated with a single dose of 20 Gy to the scrotal field. LC (300 mg/kg) and AMI (200 mg/kg) were given intraperitoneally 30 min before irradiation. The mean seminiferous tubule diameters (MSTDs) were calculated. Testicular damage was evaluated histopathologically using Johnsen's mean testicular biopsy score criteria. Malondialdehyde (MDA) and glutathione levels were measured in tissue samples. AMI plus RT and LC plus RT groups had significantly higher MSTDs than those in the RT group (p = .003 and p = .032 respectively). MDA values of both AMI plus RT and LC plus RT groups were significantly lower than those in RT group (p < .004 and p < .012 respectively). As a result, AMI and LC have a similar radioprotective effect against radiation-induced acute testicular damage, histopathologically and biochemically.

KEYWORDS

amifostine, L-carnitine, radiotherapy, testis

1 | INTRODUCTION

One of the most important components of the multimodal care of patients with cancer is external beam radiotherapy (RT). However, radiation-induced damage to the normal tissues is a dose-limiting factor that reduces the effectiveness of radiation (Baliga & Rao, 2010).

Diagnostic and therapeutic uses of ionising radiation have adverse effects on the reproductive system. Although the testes are generally not directly irradiated in the clinical setting, they receive a significant amount of scatter radiation when the pelvis or abdomen is irradiated, potentially resulting in infertility (Kim et al., 2011). The scattered dose to the testes is approximately 1%–2% of the total dose applied to the tumour during abdominopelvic RT (Budgell, Cowan, & Hounsell, 2001). The effects of ionising radiation are mainly mediated through the free radicals generated from the radiolytic decomposition of cellular water, including superoxide radicals, hydrogen peroxide and hydroxyl radicals, and all of which can cause damage to most major cellular macromolecules, such as DNA, proteins and lipids, resulting in cellular dysfunction and mortality.

A major factor in the aetiology of male infertility is oxidative stress (Sharma, Parmar, Verma, & Goyal, 2011). Therefore, a reduction in the severity of acute injuries experienced during RT might be a potential therapeutic strategy to limit the consequential late adverse effects of radiation. Prophylactic use of radioprotectants prior to fractionated irradiation can be an effective strategy for the reduction of testicular damage.

Amifostine (AMI) (Ethyol; WR-2721) is a prodrug converted by alkaline phosphatase to an active sulphydryl compound (WR-1065). WR-1065 selectively protects normal cells against irradiation and antineoplastic drug toxicity by scavenging free radicals, donating

hydrogen ions to free radicals, depleting oxygen and binding to active derivatives of antineoplastic agents (Chatal & Hoefnagel, 1999; Levi et al., 2002). The effectiveness of AMI as a radioprotective agent in an irradiated testis has been demonstrated in previous studies (Andrieu et al., 2005; Jaimala & Pareek, 1984).

L-carnitine (3-hydroxy-4-trimethylammoniumbutyric acid) (LC) is a small water-soluble molecule that facilitates the transfer of long-chain fatty acids into the mitochondria, where they undergo beta-oxidation (Vanella et al., 2000). LC decreases damage to the cell membrane by preventing the formation of reactive oxygen species produced by the xanthine/xanthine oxidase system (Bertelli, Conte, & Ronca, 1994). LC and acetyl-LC (ALC) play a crucial role in sperm metabolism and maturation and are highly concentrated in the epididymis (Agarwal & Said, 2004). Additionally, protective effect of LC on testicular tissue against radiation-induced damage has been shown in several previous studies (Amendola et al., 1989; Kanter, Topcu-Tarladacalisir, & Parlar, 2010; Topcu-Tarladacalisir, Kanter, & Uzal, 2009).

We hypothesised that LC might have a radioprotective activity in testicular tissue comparable with AMI. Therefore, the aim of this study was to use biochemical and histopathological methods to determine and compare the radioprotective effects of AMI and LC against radiation-induced testicular toxicity. To the best of our knowledge, this study was the first time this topic has been studied in the literature.

2 | MATERIALS AND METHODS

2.1 | Animals

The experimental protocol was approved by the Ethical Committee of Trakya University Medical Faculty. In this study, 30 healthy male Wistar albino rats, weighing 200–250 g and averaging 16 weeks old, were utilised. The rats housed at the Trakya University Animal Care and Research Unit were used for this study. Food and tap water were available ad libitum. In the windowless animal quarter, automatic temperature (21 ± 1°C) and lighting controls (12-h light/12-h dark cycle) were performed. The relative humidity ranged from 55% to 60%. All animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.

2.2 | Experimental design

The 30 male rats were randomly divided into four groups:

1. Control group (n = 6): rats pretreated with 1 ml 0.9% NaCl solution with a single-dose injection intraperitoneally (i.p.) without radiation.
2. AMI + RT group (n = 8): rats pretreated with 1 ml AMI (200 mg/kg) (Ethyol; MedImmune Pharma B.V., Nijmegen, the Netherlands) with a single-dose injection i.p., 30 min prior to irradiation and then irradiated individually with a single dose of 20 Gy radiation.
3. LC + RT group (n = 8): rats pretreated with 1 ml LC (300 mg/kg) (Santa Pharma Co., Istanbul, Turkey) with a single-dose injection i.p., 30 min prior to irradiation and then irradiated individually with a single dose of 20 Gy radiation.
4. RT group (n = 8): rats pretreated with 1 ml 0.9% NaCl solution i.p., 30 min prior to irradiation and then irradiated individually with a single dose of 20 Gy radiation.

2.3 | Scrotal irradiation

The rats in the AMI + RT, LC + RT and RT groups were irradiated individually with a single dose of 20 Gy. The rats were anaesthetised and then fixed onto a 20 × 30 cm blue Styrofoam treatment couch (Med-Tec, Orange City, IA, USA) in a supine position. Correct positioning of irradiation fields was controlled for each rat using a therapy simulator (Mecaserto-Simics, Paris, France). Irradiation was delivered by a cobalt-60 teletherapy unit (Cirus; cis-Bio Int., Gif Sur Yvette, France) at a source–surface distance of 65 cm. A single dose of 20 Gy radiation was given at a depth of 1.5 cm (half thickness) with a dose rate of 1.05 Gy/min to the 5 × 5 cm scrotal area. Special dosimetry was used for the irregular fields. The dose homogeneity across the field was ±5%. After the irradiation of both testes, the animals were closely observed until recovery from anaesthesia. The animals in the control group received an equal-field sham irradiation.

2.4 | Euthanasia

The rats were euthanised 5 days after the radiation therapy. Prior to euthanasia, the rats received anaesthesia using a combination of ketamine and xylazine. Euthanasia was performed by decapitation.

2.5 | Biochemical analysis (malondialdehyde and glutathione assays)

The right testis tissue samples were homogenised with 150 mmol/L ice-cold KCl for the determination of malondialdehyde (MDA) and glutathione (GSH) levels. Homogenates were centrifuged at 2,600 g for 10 min at 4°C. The MDA concentrations in the testis tissue, which are an indicator of lipid peroxidation, were assayed in the form of thiobarbituric acid-reacting substances (Ohkawa, Ohishi, & Yagi, 1979). Supernatant (200 ml) was added to 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of 0.8% thiobarbituric acid and 0.6 ml of distilled water. This mixture was heated to 95°C for 60 min. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of a mixture of n-butanol:pyridine (15:1, v/v) was added, and the mixture was shaken vigorously and centrifuged at 2,600 g for 10 min at 25°C. The absorbance of the organic layer was read at 532 nm. MDA was quantified using an extinction coefficient of 1.56×10^5 L mol^{-1} cm^{-1} and is expressed as nmol MDA/mg tissue.

The GSH level was determined according to the method of Ellman (Ellman, 1959). The concentration of GSH was monitored

TABLE 1 Testicular MDA and GSH levels are summarised for each group

Groups	Control median (range: min–max)	amifostine (AMI) + RT median (range: min–max)	l-carnitine (LC) + RT median (range: min–max)	radiotherapy (RT) median (range: min–max)	*p* Value
MDA (nmol/mg)	0.041 (0.03–0.05)	0.047 (0.03–0.05)	0.036 (0.02–1.08)	2.846 (0.06–4.13)	**<.05**
GSH (μmol/g)	3.52 (2.51–4.51)	3.25 (2.63–4.10)	3.68 (2.84–4.33)	4.42 (2.98–5.34)	**>.05**

AMI, amifostine; LC, l-carnitine; RT, radiotherapy; MDA, malondialdehyde; GSH, glutathione; *p* value: Kruskal–Wallis. Values are medians with ranges. <.05 was considered statistically significant.

spectrophotometrically at 412 nm. Results are expressed as μmol/g tissue.

2.6 | Histopathological evaluation

The left testis of each rat was cut longitudinally and fixed in a 10% formalin solution for 24 hr. They were dehydrated in alcohol and embedded in paraffin. Five micrometre sections were obtained, deparaffinised and stained with haematoxylin and eosin. The testicular tissue was evaluated in a random order with Zeiss Axioplan-2 Imaging light microscopy (KS-300 Imaging System) by a pathologist, who was blinded to the study group allocations. The mean seminiferous tubule diameter (MSTD) was measured in μm. Spermatogenesis was assessed histopathologically using Johnsen's mean testicular biopsy score (MTBS) (Johnsen, 1970). A score of 0–10 (a score of 0 means no epithelial maturation and a score of 10 means full epithelial maturation) was given to each tubule according to its epithelial maturation.

2.7 | Statistical analysis

The Kruskal–Wallis test was used to assess the statistical significance of the comparisons. Normal distributions of groups were checked. Results were expressed as median (minimum–maximum). The MDA and GSH values of the groups were compared using the Mann–Whitney *U* test with a Bonferroni correction. Comparisons of seminiferous tubule diameter and Johnsen's scores between groups were made using an ANOVA test. The results are expressed as mean ± standard deviation, and *p* values below .05 were considered to be statistically significant.

3 | RESULTS

3.1 | Biochemical analysis

The testicular MDA and GSH values in all groups are shown in Table 1. MDA levels were significantly different between all groups ($p < .05$). The levels of testicular tissue MDA were significantly higher in the RT group when compared with those in control group ($p < .006$). The levels of testicular tissue MDA were significantly lower in the AMI + RT group when compared with the RT group ($p < .004$). Additionally, the levels of testicular tissue MDA were significantly lower in the LC + RT group when compared with the RT group ($p < .012$). The differences in the levels of testicular tissue MDA were not significant between the AMI + RT group and the LC + RT group ($p > .05$). The differences in the testicular tissue GSH levels were not significant between all groups ($p > .05$).

3.2 | Histopathological evaluation

Histopathological analyses were made on 30 rats. The main radiation-induced histopathological finding was Johnsen's MTBS. Table 2 shows the MSTD and Johnsen's MTBS values for the testes in each group.

TABLE 2 Mean seminiferous tubule diameter (MSTD) and Johnsen's mean testicular biopsy score (MTBS)

Groups	mean seminiferous tubule diamete (MSTD) (μm)	*p* Value	Johnsen's mean testicular biopsy score (MTBS)	*p* Value
Control versus RT	21.28 ± 14.88	.165	1.53 ± 0.18	**<.0001**
Control versus intervention (AMI + RT)	35.78 ± 14.88	**.024**	0.95 ± 0.18	**<.0001**
RT versus intervention (AMI + RT)	14.5 ± 13.77	.302	0.57 ± 0.17	**.003**
Control versus intervention (LC + RT)	17.77 ± 14.88	.243	1.13 ± 0.18	**<.0001**
RT versus intervention (LC + RT)	3.50 ± 13.77	.801	0.39 ± 0.17	**.032**
Intervention (AMI + RT) versus intervention (LC + RT)	18.01 ± 13.77	.203	0.18 ± 0.17	.296

AMI, amifostine; LC, L-carnitine; RT, radiotherapy; MSTD, mean seminiferous tubule diameter; MTBS, Johnsen's mean testicular biopsy score.
Values are mean ± *SD*. <.05 was considered statistically significant.

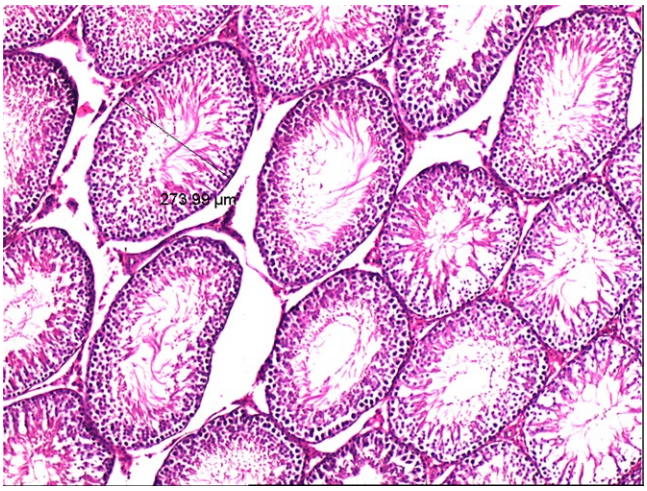

FIGURE 1 Control group showed the presence of normal testicular architecture and regular seminiferous tubular morphology with normal spermatogenesis and the presence of primary and secondary spermatocytes, spermatids and spermatozoa (H&E ×50)

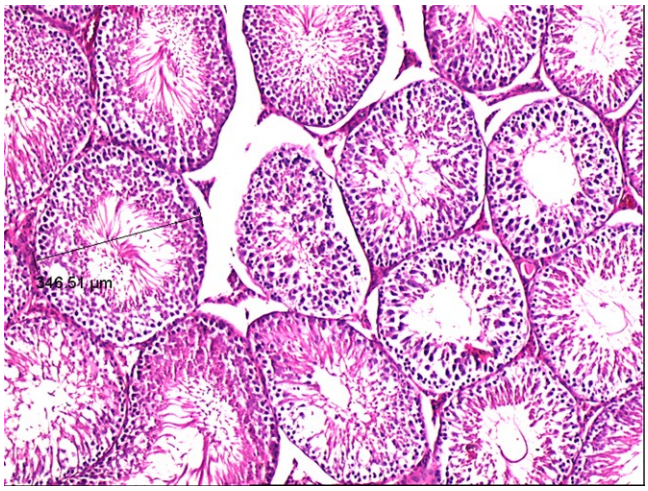

FIGURE 2 Radiotherapy group showed marked arrest in spermatogenesis, disorganisation in stratification of spermatogenic cells, occurrence of rare multinucleated giant cells and vacuolisation in the germinal epithelium (H&E ×50)

Johnsen's MTBS was significantly lower in the RT group when compared with those in the control group ($p < .0001$). Additionally, Johnsen's MTBS was significantly lower in the AMI + RT group when compared with the control group ($p < .0001$). Johnsen's MTBS was significantly higher in the AMI + RT group when compared with the RT group ($p = .003$). Johnsen's MTBS was significantly lower in the LC + RT group when compared with the control group ($p < .0001$). Johnsen's MTBS was significantly higher in the LC + RT group when compared with the RT group ($p = .032$). The difference in Johnsen's MTBS between the AMI + RT group and the LC + RT group was not significant ($p = .296$). The MSTD values were not significantly different between all groups ($p > .05$).

The findings of the histopathological evaluation for each group were considered. The testes of the rats in the sham-operated control group showed the presence of normal testicular architecture and regular seminiferous tubular morphology with normal spermatogenesis and the presence of primary and secondary spermatocytes, spermatids and spermatozoa (Figure 1).

Morphologically, as compared to the nonirradiated testes, the examination of irradiated testes revealed the presence of marked arrest in spermatogenesis, disorganisation in the stratification of spermatogenic cells, the occurrence of rare multinucleated giant cells and vacuolisation in the germinal epithelium (Figure 2).

Coadministration of AMI with irradiation reduced the histopathological changes and decreased spermatogenesis damage, and less disorganisation in the stratification of spermatogenic cells was seen. Additionally, the emergence of multinucleated giant cells and vacuolisation decreased in the germinal epithelium after AMI treatment. Coadministration of LC with irradiation reduced the histopathological changes and decreased spermatogenesis damage, and less disorganisation in the stratification of spermatogenic cells was seen. Additionally, the emergence of multinucleated giant cells and vacuolisation decreased in the germinal epithelium after LC treatment.

4 | DISCUSSION

The main findings of our study were as follows: (i) a marked radiation-induced acute testicular toxicity was seen 5 days after with a single dose of 20 Gy; (ii) both AMI and LC have similar radioprotective effects on testicular tissue against all histopathological changes; and (iii) these protective effects can be demonstrated with both biochemically and histopathologically.

Although ionising radiation is commonly applied in medicine, such as for imaging and therapeutic reasons, data about its acute and late adverse effects on testicular tissue have been limited. Ionising radiation mostly affects rapidly growing cells. In testicular tissue, the most radiosensitive cell types are spermatogonia. Morphologically, ionising radiation causes the arrest of spermatogenesis, desquamation, vacuolisation of germinal cells and the emergence of multinucleated giant cells. After irradiation, spermatogonia cells are rapidly depleted, and their survival rate is the most important determining factor of future fertility.

The type and extent of radiation-induced morphological changes are related to the dose, duration and frequency of irradiation (Bansal, Kaul, Tewari, & Nehru, 1990; Hussein, Abu-Dief, Abou El-Ghait, Adly, & Abdelraheem, 2006). Bansal et al. (1990) performed a quantitative histologic evaluation of the testes of albino rats that were exposed to scrotal γ-irradiation doses of 2.5 and 10.0 Gy. Testicular weight decreased and the seminiferous tubules showed arrest of spermatogenesis, germinal cell vacuolisation and multinucleated giant cell formation. Increasing posttreatment time interval and the dose maintained the extent of damage. This study revealed that the pre-leptotene spermatocyte is the most sensitive to radiation. Pinon-Lataillade, Viguier-Martinez, Touzalin, Maas, and Jegou (1991) mentioned that after acute exposure of 2-month-old rat testes to 9 Gy of gamma rays, spermatogonia and preleptotene spermatocytes that were replicating their DNA at the time of exposure were mainly affected. Additionally,

Sertoli cells were not affected after irradiation when compared with the control group. Highly differentiated Sertoli cells are radioresistant, and previous studies indicated that Sertoli cells were not affected after irradiation (Guitton, Brouazin-Jousseaume, Dupaix, Jegou, & Chenal, 1999; Jegou et al., 1993). However, some previous studies described minimal changes in Sertoli cells (Kangasniemi, Huhtaniemi, & Meistrich, 1996; Shuttlesworth et al., 2000).

Radiation-induced testicular damage starts only hours after irradiation. In a study performed by Lee et al. (2008), germ cell apoptosis increased at 2 hr after irradiation, and the maximum effect was observed at 12 hr. Hussein et al. (2006) assessed the protective effect of melatonin in the testicular tissue of rats 48 hr after irradiation, observing a marked depletion of spermatogenic cells. In a pioneering study, Eschenbrenner and Miller (1950) observed that 7 days after irradiation, there was an absence of spermatogonia in mouse testes. In another study, Nebel and Murphy (1959) stated that the greatest increase in restitution nuclei seemed to occur between 2 and 4 days after irradiation and reached the maximum number at the sixth day. They also stated that tubular shrinkage can be observed up to 2 weeks after irradiation, as recovery is initiated at that time. As we aimed to assess the protective effects of AMI and LC on acute radiation-induced testicular damage, based on the above-mentioned studies, we studied rat testicles 5 days after irradiation.

The radiation dose to the pelvic or testicular area in previous studies ranged widely—from 3.5 Gy to 60 Gy. While Kangasniemi et al. (1996) used doses of 3.5 and 6 Gy, Milas, Hunter, Reid, and Thames (1982) used 14 Gy and Gupta and Bawa (1975) used doses ranging from 7.2 Gy to 60 Gy. In one of the earliest studies on the effects of testicular radiation, Fogg and Cowing (1952) found that more than 20 Gy is necessary for permanent sterility in a male mouse. In the present study, we gave doses of 20 Gy to the testes to yield marked acute histopathological changes similar to these studies. After histopathological evaluation, we observed arrest in spermatogenesis, disorganisation in the stratification of spermatogenic cells, the occurrence of rare multinucleated giant cells and vacuolisation in the germinal epithelium. Sertoli cells were not affected by irradiation with a single dose of 20 Gy, which was expected.

Amifostine (Ethyol; WR-2721) is an organic thiophosphate ester prodrug and must be activated by alkaline phosphatase to be converted into an active sulfhydryl compound (WR-1065) (Chatal & Hoefnagel, 1999; Levi et al., 2002). WR-1065 selectively protects normal cells against irradiation and antineoplastic drug toxicity by scavenging free radicals, donating hydrogen ions to free radicals, depleting oxygen and binding to active derivatives of antineoplastic agents (Kouloulias et al., 2004).

There have been several experimental studies investigating the radioprotective role of AMI on testicular tissue in animals. The authors of these studies used several methods for this purpose. Similar to our study, Jaimala and Pareek (1984), Milas et al. (1982) and Andrieu et al. (2005) used also a histopathological assessment of testes.

Jaimala and Pareek (1984) assessed the protective role of AMI on spermatogonia in mice against different doses of radiation. They observed the notable protective potential of AMI against radiation. In that study, the cell counts were constantly higher in the radiation with AMI groups than the radiation without AMI groups. Milas et al. (1982) investigated the protective effect of AMI against acute radiation-induced damage on several tissues such as the jejunum, the testes, the lungs, hair follicles and a fibrosarcoma of C3Hf/Kam mice. They observed that testicular tissue was more protected if more drug was given to animals. Moreover, testicular tissue was maximally radioprotected at ten min. They concluded that radioprotective effects of AMI depended on the dose of the compound and the time interval between the administration of the compound and the radiation treatment. Finally, Andrieu et al. (2005) ultrastructurally and histologically investigated the radioprotective effect of AMI on rat testes. They found that pretreatment with AMI significantly reduced the decrease in primary spermatocyte counts but not spermatogonia counts. Additionally, the authors did not observe any protective effects AMI on the Johnsen's score. However, in our study, Johnsen's MTBS was significantly higher in the AMI + RT group than the RT group ($p = .003$). Therefore, we observed that AMI pretreatment had radioprotective effects on spermatogenesis, protecting epithelial maturation. We saw that the histopathological changes were reduced and spermatogenesis damage was decreased with AMI. The emergence of multinucleated giant cells and vacuolisation were also decreased in the germinal epithelium. Additionally, unlike these studies, this protection was also supported by biochemical analysis. In the presented study, while testicular tissue MDA levels were significantly high in the RT group ($p < .006$), pretreatment with AMI yielded significantly lower MDA levels when compared with the RT group ($p < .004$).

L-carnitine is a substance that can act as an antioxidant and free radical scavenger (Rolleman et al., 2007). Additionally, LC has the capacity to control carbohydrate metabolism and to maintain cell membrane structure and cell viability, and it is an essential cofactor in the oxidation of long-chain fatty acids (Caloglu et al., 2009). It protects the lipids of biological membranes against oxidation and consequently inhibits the accumulation of lipid peroxidation products, such as MDA, after testicular ischaemia-reperfusion injury in rats (Dokmeci et al., 2007). Additionally, its protective effect on testicular tissue against radiation-induced damage has been histopathologically and ultrastructurally investigated in several previous studies using oxidative damage parameters, but this effect has never before been biochemically investigated with these parameters.

Amendola et al. (1989) administered i.p., (100 mg/kg) LC and investigated the influence of it on the recovery processes of mouse spermatogenesis after local acute irradiation with 10 Gy X-rays. The ALC-treated animals showed a faster recovery from tetraploid spermatids to round and elongated spermatids and a positively affected maturation process. Their results indicated that the presence of exogenous ALC could enhance the recovery of spermatogonial cells.

Kanter et al. (2010) administered (200 mg/kg, i.p.) LC and evaluated the effects of LC on the apoptosis of germ cells in rat testes following a single dose of 10 Gy scrotal γ-irradiation. Their results underlined that the pretreatment of rats with LC 24 hr before exposure to γ radiation significantly attenuated the radiation-induced histopathological changes and germ cell apoptosis. Additionally, ionising

radiation caused no statistically significant alterations in the MSTD 24 hr after irradiation, which is consistent with our study. In a previous work of these authors, LC (200 mg/kg, i.p.) was administered one day before 10 Gy scrotal γ radiation and 21 days (three times per week) after irradiation. Testis samples of all the study groups were taken at 21, 44 and 70 days after irradiation. The histopathological examination revealed depletion of germ cells, arrest of spermatogenesis and germinal epithelial vacuolisation. At day 21, testicular damage was evident, reaching the highest level by day 44 (Topcu-Tarladacalisir et al., 2009).

In the present study, LC pretreatment also showed protective effects on spermatogenesis comparable to AMI. Histologically, LC protected epithelial maturation. The occurrence of multinucleated giant cells and vacuolisation were also decreased in the germinal epithelium. Furthermore, the decrease in Johnsen's MTBS with irradiation was improved by LC pretreatment. Johnsen's MTBS was significantly higher in the LC + RT group compared with the RT group ($p = .032$). Additionally, the protective effect of LC was also supported by biochemical analysis that showed that LC significantly decreased MDA levels to control levels after irradiation ($p < .012$). This protection was similar to the protection yielded by AMI; there was no difference between the Johnsen's MTBS of the two groups ($p = .296$) and the levels of testicular tissue MDA of the two groups ($p > .05$).

5 | CONCLUSION

The findings of this study showed that AMI and LC have similar radioprotective effects on testicular tissue. To the best of our knowledge, this study was first time that biochemical analysis, specifically the determination of MDA and GSH levels, was used alongside histopathological findings to detect the protective effects of AMI and LC after RT. Additionally, this study is the first in which the protective effects of LC was compared to those of AMI. However, based on the experimental results of this study, further investigations are warranted and additional human studies are required to clarify the protective role of AMI and particularly LC on radiation-induced acute and chronic testicular toxicity.

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